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13. ABSTRACT (Maximum 200 Words)
Tubulin, a potential target for chemotherapy, plays an important role in different cellular functions including mitosis and cell division. Tubulin consists of mainly α and β subunits: each subunit exists in several isoforms. The distribution and expression of β-isoforms differ from tissue to tissue: β_{II} and β_{IV} are ubiquitously expressed while β_{III} expression is restricted to neurons and cancer cells. Moreover, $\alpha\beta_{III}$ has least affinity for anti-tubulin drugs compared to $\alpha\beta_{II}$ and $\alpha\beta_{III}$ tubulin. In this study, we correlated the expression of β_{III} tubulin and oxidative stress related to drug resistance in cancer cells. We have established a taxol-resistant BT474 breast cancer cell line (IC_{50}= 0.97 nM) from the taxol-sensitive line of BT454 (IC_{50}=0.5 nM). We found that taxol-resistant BT454 cell line experienced higher oxidative stress compared to its sensitive line and the expression of β_{III} tubulin was also found significantly elevated in drug-resistant cells. However, the expression of actin and β_{II} tubulin remained unchanged. These data therefore, strongly suggest that selective elevation of expression of specific β_{III}-tubulin cause development of drug resistance in breast cancer cells and the elevated level of formation of reactive oxygen species might be directly related to drug resistance.

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Introduction

Tubulin is a major and potential target for chemotherapy for breast and other cancers. Tubulin is a $\alpha\beta$ heterodimeric protein and a major structural component of microtubules that play critical roles in mitosis: both α and β tubulin exists in several isoforms that are encoded by different genes. Among the isoforms, the β-tubulin isoforms are the most diverse in their sequences especially at their C-terminal regions. All these tubulin isoforms are expressed differently: β_{II} and β_{IV} are widespread in all our tissues while β_{III} has a restricted distribution (neuron) although it is elevated in many tumors. The ideal drug for therapy would be the one that would selectively bind to the heterodimer of β_{III}, $\alpha\beta_{III}$. Unfortunately, most successful anti-tumor drugs such as Taxol derivatives and *Vinca* alkaloids that are routinely used for the treatment of cancer bind least well to $\alpha\beta_{III}$ and best to either $\alpha\beta_{II}$ or $\alpha\beta_{IV}$. Not surprisingly, the breast cancer cells treated with these drugs increase the expression of β_{III} tubulin that causes drug resistance and creates a major obstacle in treating cancers. In our previous report, we demonstrated that the breast and other cancer and drug-resistant cells had elevated level of β_{III} tubulin with increased levels of reactive oxygen species (ROS) compared to normal cell. Therfore, the major objective in this proposal was to establish a direct relationship between β_{III} tubulin expression, drug resistance and the oxidative stress in cancer cells. We hypothesize that cancer and drug resistant cells elevate the expression of β_{III} tubulin to protect microtubules from the oxidative stress generated by anti-tubulin drugs. The over- expression of β_{III}-tubulin that has lower affinity for anti-tumor drug, such as taxol, causes the development of drug resistance. The result of this work will help in understanding the importance and role of the β_{III} isoform and the reactive oxygen species in the progression of drug resistance in breast cancer cells.

Body:
The central hypothesis of this proposal is to test that the development of drug resistance by breast cancer cells is accompanied by increased expression of the β_{III} isoform and increased oxidizing conditions in the cells.

The statement of the work for the second year (6/1/03 to 5/30/04) is described below:

A. We will develop breast cancer drug-resistant cell line against taxol.

We have taken BT474 breast cancer cell line in developing drug resistance cell line against taxol. BT474 cultured in DMEM/F12, 10% PBS, 0.05% pen/strep, was exposed continuously to taxol (0.98 nM) in every passage of transfer. We used P-glycoprotein inhibitor, verapamil, to make sure that the artificial development of drug resistance was not due to simply expression of P-glycoprotein. The IC_{50} for BT474-resistant cell was 0.97 nM compared to BT474-sensitive ($IC_{50} = 0.5$ nM). Although the IC_{50} was only increased by two fold, the morphology of the drug-resistant cell (**Figure 1A**) was distinctly different from the BT474-sensitive cell line (**Figure 1B**) which suggested that drug-resistant cells went through a significant alteration in their structures to adapt the artificial toxic environments.

B. After we establish the drug-resistant cell lines, we will measure the expression of different β-tubulin isoforms and the level of reactive oxygen species. We will also grow normal and different established breast cancer cell lines to measure the level of expression of β-tubulin and its different β-tubulin isoforms namely β_{II} and β_{III}. We will also measure the level of reactive oxygen species in normal and cancer cell lines.

The monoclonal antibodies JDR.3B8 and SDL.3D10 specific, respectively, for the β_{II} and β_{III} isoform of tubulin were prepared as previously described (1,2). The peptides used as immunogens for raising antibodies were CEGEEDEA and CESESQGPK, respectively for β_{II} and β_{III} tubulin. Immunoblotting of gels was carried out as previously described (2) except that the secondary antibody, instead of being radioactive, was conjugated with horseradish peroxidase. In brief, BT474 taxol-sensitive and resistant cell lines, normal (A-10), breast (MCF-7, MDA435 and BT5492) and other cancer cells (PC-3, DU145, HeLa, Hs578T) and a transformed cell (HUVEC) grown on Petri dishes were lysed with ice cold buffer, containing 20 mM Tris-HCl, pH 7.5, 1% sodium dodecyl sulfate, 2 mM EGTA and complete proteinase inhibitor cocktail tablets and sonicated by 30 sec cycles. Protein concentration was determined by Lowry before polyacrylamide gel electrophoresis (PAGE) carried out in 7.5% SDS-discontinuous gels. 30 µg of protein extract was loaded in each lane of the gel. Proteins were electrotransferred to nitrocellulose membranes, which were stained with Ponceau S to verify the amount of protein transferred in each lane. The nitrocellulose paper was blocked with 5% skim in PBS and incubated in a 1:10,000 dilution of the $\beta_{II,}$ 1:5000 dilution of the β_{III} antibody and 1:10,000 for actin antibody overnight at 4 °C. The blots were washed at least three times (20 min each) in PBS and incubated for 1 hr in a 1:5000 dilution of peroxidase-

conjugated goat anti-mouse IgG. Positive bands were visualized with the Amersham enhanced chemiluminiscence ECL kit. The protein concentration was measured by method of Lowry et al (3). In **Figure 2**, we first measured the expression of actin , β_{II} and β_{III} tubulin isoforms in BT474 taxol-sensitive and resistant cell line. **Figure 2A** showed the expression of actin, **Figure 2B** showed the expression of β_{II} and **Figure 2C** represented the expression of β_{III}. We found that the expression of actin and the β_{II} tubulin was not changed with the development of drug resistance against taxol. However, the expression of β_{III} tubulin in drug resistant cell was elevated significantly compared to the sensitive cell line. These data strongly suggested that the significant elevation of expression of β_{III} tubulin in BT474 breast cancer cell caused the development of resistance to taxol.

In **Figure 3**, we also tested the level of expression of β, β_{II} and β_{III} tubulin isoforms in nontransformed and cancer cells. The top panel showed the expression of β-tubulin in different cell lines. (+) was the $\alpha\beta$ tubulin that was used for positive control and rests were the extracts from different cell lines (A10, BT549L, DU145, HeLa, Hs578t, HUVEC, MCF-7, MDA-MB-435 and PC-3). The middle panel showed the expression of βII in different cell lines. As $\alpha\beta_{II}$ constitutes 52% in unfractionated $\alpha\beta$ tubulin and is a major isoform expressed ubiquitously in all tissues, we detected significant amount of β_{II} in all cancer as well as in A-10 cells (smooth muscle) that is considered as normal cells. Interestingly, we did not detect the β_{III} tubulin in A-10 but detected in all breast and other cancer cell lines supporting the earlier observations (4-7) that the expression of β_{III} tubulin is restricted to transformed and cancer cells. Moreover, we detected 29 times more β_{III} in BT5492 compared to MDA435.

We measured the level of reactive oxygen species (ROS) in BT474 taxol-sensitive and resistant cells to examine the direct relationship between drug resistance and oxidized state **(Figure 4)**. We also measured the level of ROS (reactive oxygen species) for normal smooth muscle, breast and other cancer cell lines namely, A-10, MCF-7, HeLa, BT549L and PC3 **(Figure 5)**. DCFA (dichlorofluorescein diacetate) was used as a probe in measuring the level of intracellular ROS. The excitation and emission wave length for DCFA was 504 and 524 nm, respectively. In brief, after cells grew in confluent, cells were treated with 20 μM DCFA for 30 min in the dark at room temperature followed by washing cells in Petri dishes with PBS at least four times. Then cells were harvested, sonicated by 30 sec cycles in presence of Pipes buffer containing 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton-X and protease inhibitors and centrifuged at 12000g at 4 °C for 30 min. The protein concentration in the supernatant of each of the sample was measured. Equal amount of protein was taken (50 μg) and the fluorescence was measured at 524 nm.

We also measured the oxidized status in normal, breast cancer and drug resistant cell lines to support the hypothesis that breast cancer and drug resistant cell lines have higher level of reactive oxygen species. I compared two breast cancer cell lines: MDA435 and BT5492 with normal smooth muscle cells (A-10). The BT5492 cells over express 29 times as much as β_{III} as do the MDA435 cells while A-10 has no

detectable β_{III} as I mentioned earlier **(Figure 3)**. When DCFA was used to measure ROS in these cells, we found a high level of ROS in BT5492 compared to MDA435. Moreover, MDA435 has much more ROS than do the A-10 cells **(Figure 6)**. Interestingly, BT5492 cells, unlike MDA435, are highly resistant to vinblastine, taxol and cryptophycin.

Key Research Accomplishments

The following observations were made from this research

1. We have established a taxol-resistant BT474 breast cancer cell line from the BT474 cell line which showed a distinct morphology compared to the sensitive one and the IC50 for the resistant and sensitive cell lines were 0.97 and 0.5 nM, respectively.
2. The level of expression of β_{III} was measured in normal smooth muscle (A-10) and in breast and other cancer cell lines to support the existing concept that β_{III} is elevated in cancer and highly elevated in drug resistant cancer cells. We found that β_{III} was not expressed in normal cell but elevated in all transformed and cancer cells, especially in breast cancer cells namely MCF-7, MDA435 and BT5492 cells. Interestingly, the level of expression was elevated maximum in BT5492 that is highly resistant to tubulin-targeted drugs such as Taxol, *Vinblastine*. This finding suggests that the expression of β_{III} and drug resistance in cancer cell is directly correlated. This observation was confirmed when the taxol-resistant BT474 breast cancer cell exhibited significant level of expression of β_{III} tubulin compared to the level of taxol-sensitive BT474 cell line.

3. We also found in general that cancer cells experience more oxidative stress compared to the normal cell. Moreover, the drug resistant cells experience maximum oxidative stress compared to other cells.

4. As cancer and drug resistant cells experience higher oxidative stress, they need structurally resistant tubulin dimer for their survival, growth and maintaining of cellular functions. The rationale for selection of $\alpha\beta_{III}$ tubulin by cancer and drug resistant cells was that the cysteines in $\alpha\beta_{III}$ tubulin are least sensitive to oxidation and $\alpha\beta_{III}$ tubulin is structurally stable than other isoforms (reported in last year progress report). In case of normal cell, the oxidative stress is markedly low compared to the cancer cells, so they do not require β_{III} tubulin for their function. That's why; normal cells do not express β_{III} tubulin.

Reportable Outcomes:
These data provide important information on the regulation of expression of β_{III} tubulin in response to exposure to anti-tubulin drug (taxol) which elevated oxidative stress in cancer and drug resistant cells. I am writing manuscript on this observation.

Conclusion:
These data all together strongly propose a probable mechanism of expression of β_{III} tubulin in breast and other cancer and drug resistant cells. I used different cancer cell lines and developed a taxol-resistant cell line to establish the fact that the observation that I reported was not restricted to breast cancer cells but rather was a general phenomenon in different cancer cells. From first and second year of our research, we found that breast cancer, other cancer (prostate) and drug resistant breast cancer cells experienced higher endogenous oxidative stress compared to the stress observed in normal smooth muscle cell (A-10). I initially proposed to use MCF-10F as normal cells. I had a hard time to have enough cells to do *in vitro* experiments. Therefore, I used A-10 to simply show that proteins in normal cells generally experience low oxidative stress compared to proteins of cancer cells and the expression of β_{III} was strictly restricted to cancer cells.

In response to high oxidative stress in cancer and drug resistant cells, β_{III} tubulin was expressed in cancer and drug resistant cells. Now question comes why cancer cells chose β_{III} tubulin in response to oxidative stress? Using surface cysteine scanning assay and the data obtained from others (8), we reported in last year annual report that $\alpha\beta_{III}$ tubulin was structurally resistant in denaturing environment as determined by exposure of the cysteine residues. Since cystines were not available on surface due to structural integrity of $\alpha\beta_{III}$ tubulin, they were not easily alkylated or oxidized by the iodoacetamide. This observation promptly suggested that the cysteines of $\alpha\beta_{III}$ tubulin would not be readily oxidized in cells even experiencing high oxidative stress. However, the cysteines of $\alpha\beta_{II}$ were more sensitive and available on surface. This unique property of $\alpha\beta_{III}$ tubulin probably assisted cancer cells to build stable microtubules to survive and maintaining cellular functions in adverse situations such as in oxidative stress.

"So what section": The data obtained from this research will provide strong evidence in understanding the function of $\alpha\beta_{III}$ tubulin in cancer and drug resistant cells. This information along with the crystal structure data will help in designing drugs that will bind tightly to $\alpha\beta_{III}$ tubulin and least to other isoforms that express ubiquitously. The positive point in designing drug against $\alpha\beta_{III}$ tubulin is that the drugs will have least cyto-toxic effect to normal cells as the expression of β_{III} is restricted to cancer and drug resistant cells.

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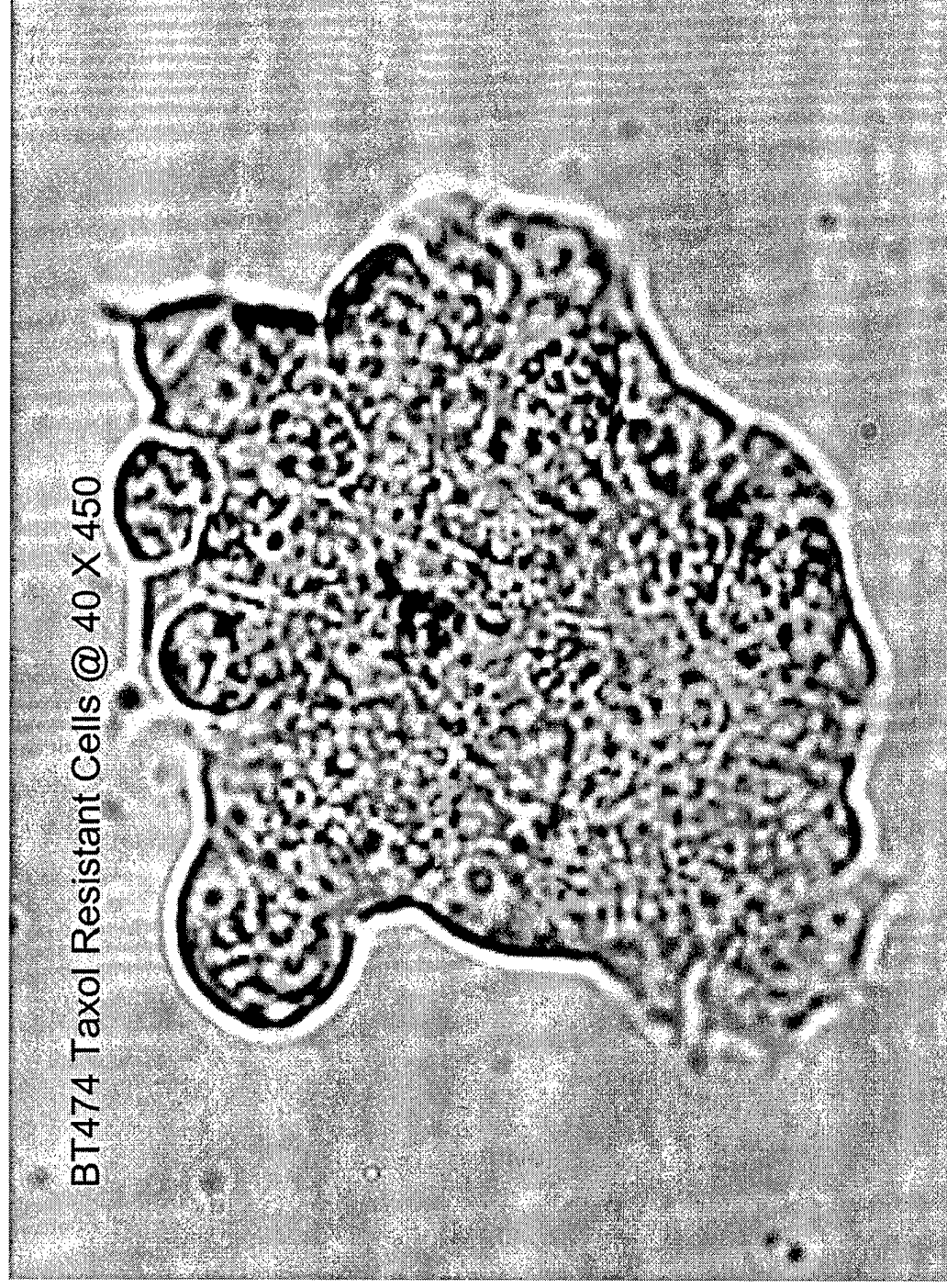# Figure 1A: Cellular Morphology of BT474 Taxol Resistant Cells

BT474 Taxol Resistant Cells @ 40 X 450

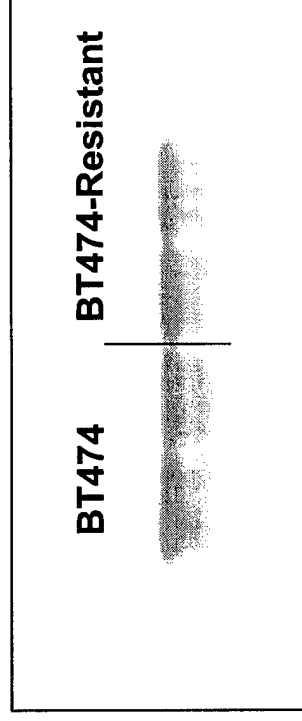# Figure 1B: Cellular Morphology of BT474 Taxol-Sensitive cell Line

BT474 Regular Cells @ 40 X 436
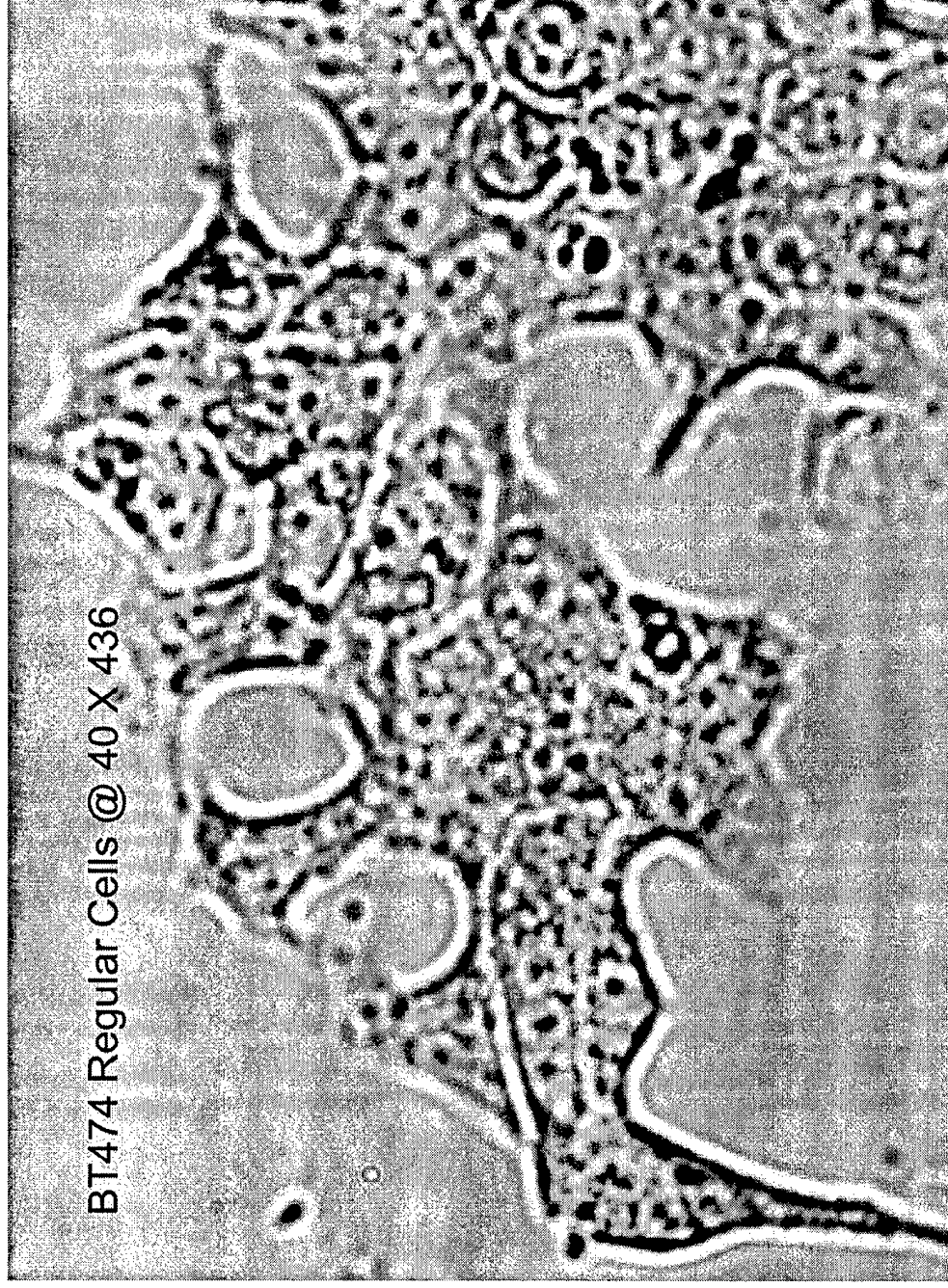
Figure 2A: Western Blot Analysis of the Level of Actin in Taxol-sensitive and Resistant BT474 Breast Cancer Cell Lines

BT474   BT474-Resistant

1° Actin 1:40,000 in 5% milk

2° Anti-mouse 1:20,000; peroxidase-conjugated anti-mouse antibody 1:10,000 in 5% milk

Figure 2B: Western Blot Analysis of the Level of β_{II}-Tubulin in Taxol-sensitive and Resistant BT474 Breast Cancer Cell Lines

BT474   **BT474-Resistant**
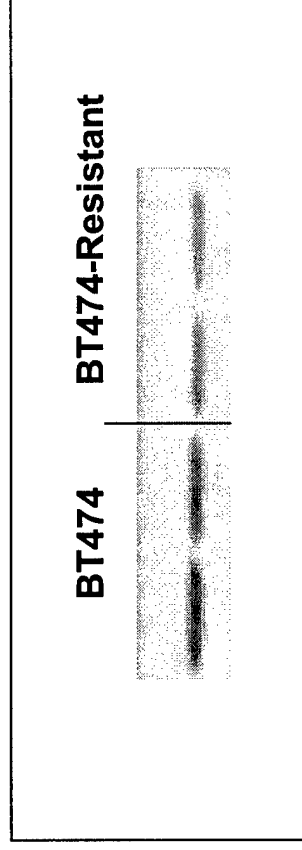
1^0 β_{II}-Tubulin 1:1,000 in 5% milk

2^0 Anti-mouse 1:10,000; peroxidase-conjugated anti-mouse antibody 1:10,000 in 5% milk

Figure 2C: Western Blot Analysis of the Level of β_{III}-Tubulin in Taxol-Sensitive and Resistant BT474 Breast Cancer Cell Lines

BT474   BT474-Resistant

1^0 Tubulin III 1:1,000 in 5% milk

2^0 Anti-mouse 1:10,000; peroxidase-conjugated anti-mouse antibody 1:10,000 in 5% milk

Figure 3: Western Blot Analysis of the Expression of β-tubulin Isoforms in Nontransformed and Cancer Cell Lines
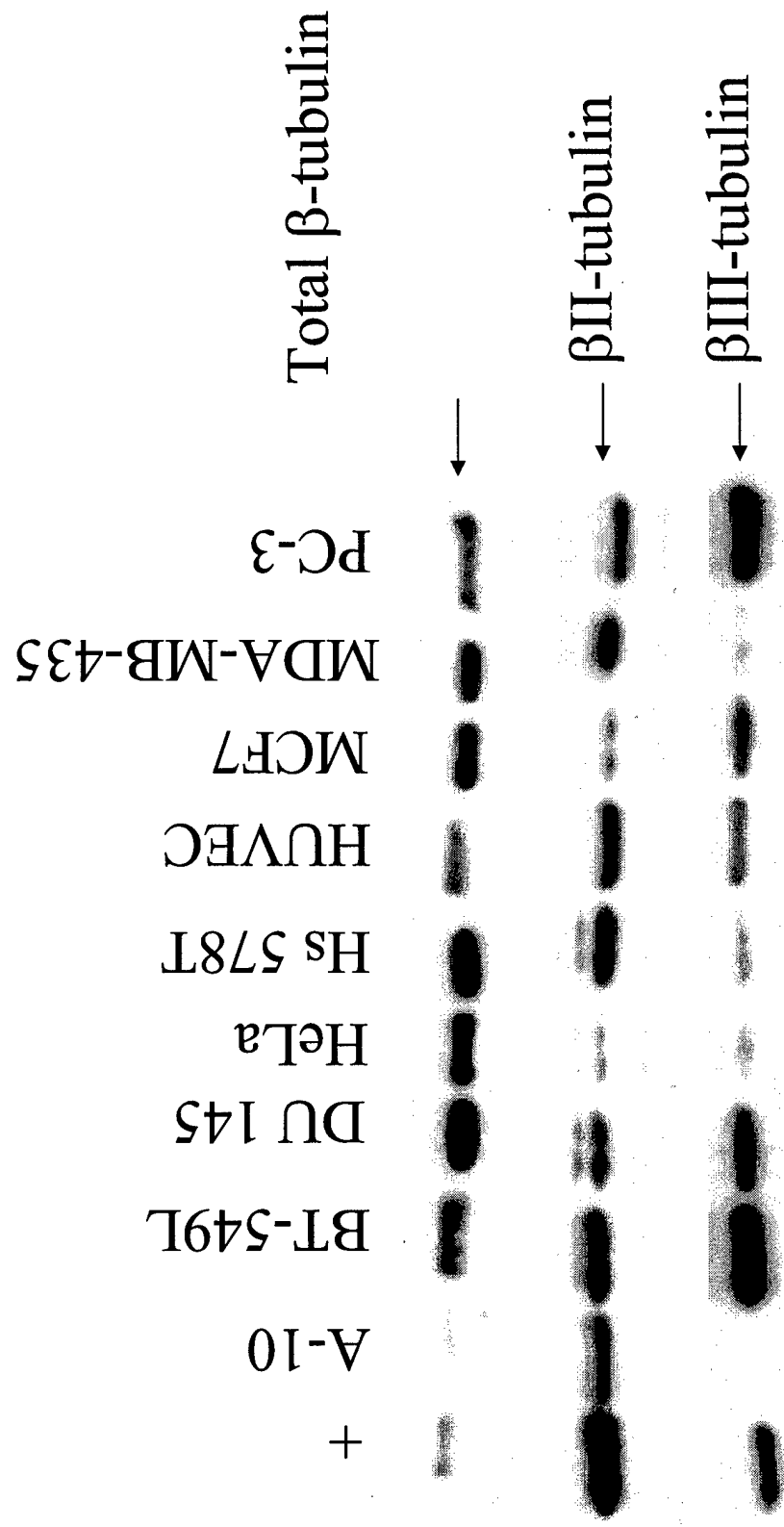
Figure 4: Measurement of the Level of ROS in Taxol-Sensitive and Resistant BT474 Breast Cancer Cell Lines
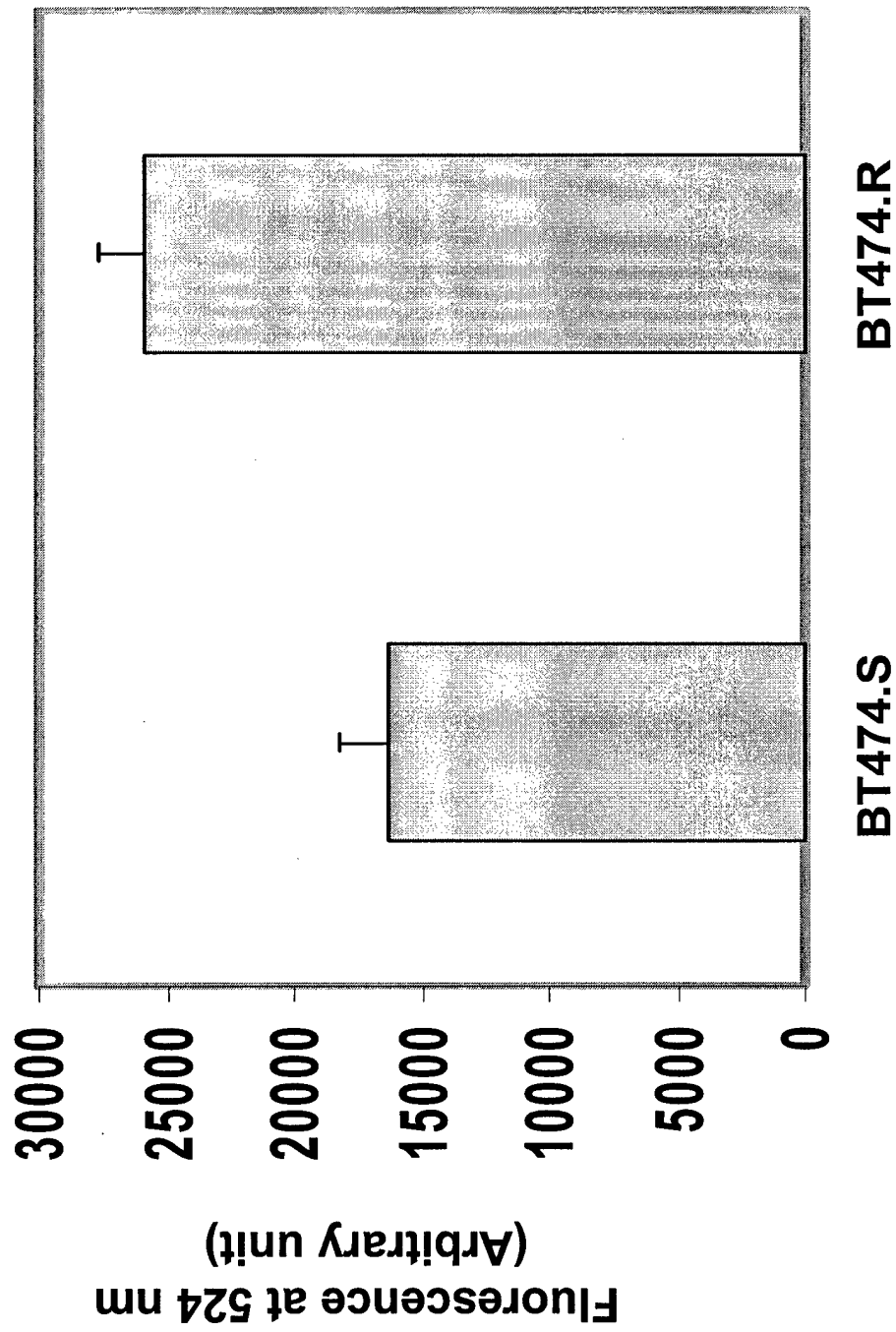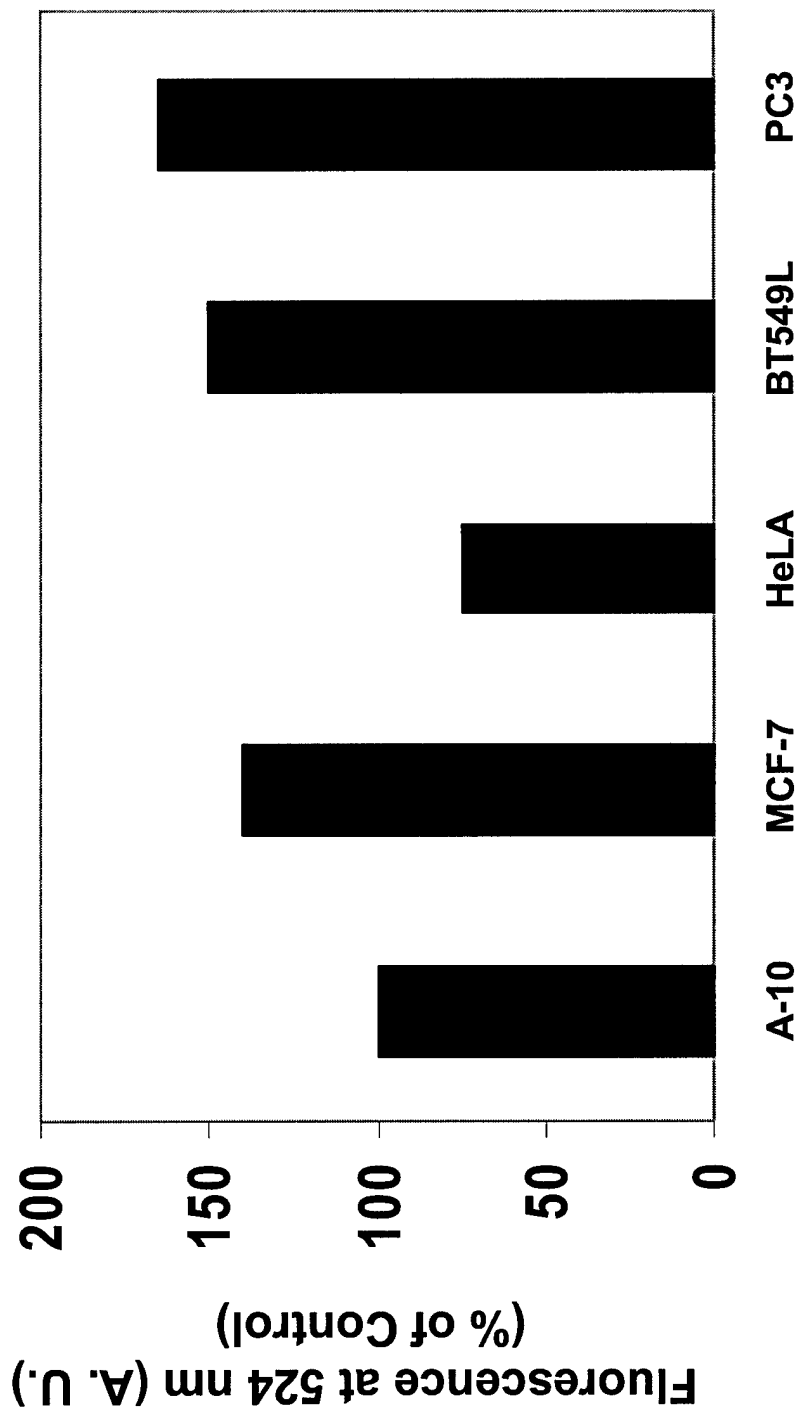
Figure 5: Measurement of the Level of ROS in Normal and Different Cancer Cell lines

Figure 6: Measurement of the Level of ROS in Normal, Breast and Drug Resistant Breast Cancer Cells

Legend:
- A-10
- MDA-435
- BT5492

Y-axis: Fluorescence at 524 nm (A.U.)
X-axis: [DCFA]

X-axis values: 0μM, 1μM, 2μM, 5μM, 10μM, 20μM

Y-axis values: 0, 100, 200, 300, 400, 500, 600, 700, 800